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The regulation of adenylate cyclase by guanine nucleotides in *Dictyostelium discoideum* membranes

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Extracellular cAMP induces the activation of adenylate cyclase in *Dictyostelium discoideum* cells. Conditions for both stimulation and inhibition of adenylate cyclase by guanine nucleotides in membranes are reported. Stimulation and inhibition were induced by GTP and non-hydrolysable guanosine triphosphates. GDP and non-hydrolysable guanosine diphosphates were antagonists. Stimulation was maximally twofold, required a cytosolic factor and was observed only at temperatures below 10°C. An agonist of the cAMP-receptor-activated basal and GTP-stimulated adenylate cyclase 1.3-fold. Adenylate cyclase in mutant N7 could not be activated by cAMP *in vivo*; *in vitro* adenylate cyclase was activated by guanine nucleotides in the presence of the cytosolic factor of wild-type but of not mutant cells.

Preincubation of membranes under phosphorylation conditions has been shown to alter the interaction between cAMP receptor and G protein [Van Haastert (1986) *J. Biol. Chem.* in the press]. These phosphorylation conditions converted stimulation to inhibition of adenylate cyclase by guanine nucleotides. Inhibition was maximally 30% and was not affected by the cytosolic factor involved in stimulation.

In membranes obtained from cells that were treated with pertussis toxin, adenylate cyclase stimulation by guanine nucleotides was as in control cells, whereas inhibition by guanine nucleotides was lost. When cells were desensitized by exposure to cAMP agonists for 15 min, and adenylate cyclase was measured in isolated membranes, stimulation by guanine nucleotides was lost while inhibition was retained. These results suggest that *Dictyostelium discoideum* adenylate cyclase may be regulated by Gs-like and Gi-like activities, and that the action of Gs but not Gi is lost during desensitization *in vivo* and by phosphorylation conditions *in vitro*.

Extracellular cAMP functions as an intercellular signal molecule in *Dictyostelium discoideum*, and is involved in chemotaxis [1], morphogenesis [2] and cell differentiation [3]. cAMP binds to highly specific cell-surface cAMP receptors, which activate several enzymes, including adenylate cyclase and guanylate cyclase (reviewed in [4–6]). The cGMP produced remains largely intracellular and is probably involved in the chemotactic reaction [7–9]. The cAMP produced is secreted [10], and may trigger more distal cells, thus relaying the signal.

The regulation of adenylate cyclase activity by cAMP in *D. discoideum* cells has been well characterized [11–14]. Enzyme activity increases at about 10–30 s after cAMP addition, reaches maximal activity after 1–2 min and then decays to prestimulation levels. The decay of adenylate cyclase activity occurs even when the cAMP concentration remains constant and proceeds with a half-life of 3–4 min. This desensitization is reversible with a similar half-life period when cAMP is

removed. Despite the detailed knowledge of the physiology of adenylate cyclase stimulation, little is known about the molecular mechanism of stimulation. Recently we observed complex binding of cAMP to *D. discoideum* cells; different interconvertible receptor forms were detected [15, 16]. The interconversions of receptor forms on cells are dependent on time and cAMP concentration. cAMP binding to isolated membranes reveals essentially the same receptor forms as binding to cells, but now interconversion of receptor forms can be induced by guanine nucleotides [16–19]. The guanine nucleotide specificity points to the action of guanine nucleotide regulatory proteins (G proteins) in cAMP receptor modulation. The existence of a G protein has been suggested previously by Leichtling et al. [20], who showed that a 42-kDa protein binds GTP and can be ADP-ribosylated by cholera toxin. In vertebrate cells G proteins mediate the signal from hormone receptor to adenylate cyclase and other effector systems. The effect on adenylate cyclase can be either stimulatory (Gs) or inhibitory (Gi) [21].

The stimulation of adenylate cyclase activity in *D. discoideum* membranes by guanine nucleotides has not been observed until now [6]. Several reasons could cause the absence of adenylate cyclase regulation *in vitro*. Firstly, it has been observed that cAMP induces a reversible covalent modification, probably phosphorylation, of the cell-surface cAMP receptor, and it has been proposed that this modification could be the molecular basis of desensitization [22–24]. In

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Abbreviations. p[NH]ppA, adenosine 5'-[β , γ -imido]triphosphate; p[CH₂]ppA, adenosine 5'-[β , γ -methylene]triphosphate; p[NH]ppG, guanosine 5'-[β , γ -imido]triphosphate; ATP[γ S] and GTP[γ S], adenosine and guanosine 5'-[γ -thio]triphosphates; GDP[β S], guanosine 5'-[β -thio]diphosphate; dcAMP 2'-deoxyadenosine 3',5'-monophosphate (Sp)-cAMP[S], (Sp)-adenosine 3',5'-[thio]monophosphate.

vertebrates it has been shown that receptor phosphorylation alters Gs-mediated stimulation of adenylate cyclase [25, 26]. In addition we recently observed that incubation of membranes under phosphorylation conditions strongly alters the interaction between the receptor and G protein in *D. discoideum* membranes [27]. Phosphorylation may take place during the adenylate cyclase assay and may affect the interaction between G protein and adenylate cyclase. Secondly, both Gs and Gi may be present in *D. discoideum* membranes; their simultaneous activation may obscure the presence of either.

In this report we have investigated the effect of temperature, Mg^{2+} concentration, phosphorylation conditions and a cytosolic factor on guanine nucleotide regulation of adenylate cyclase in *D. discoideum* membranes. Both stimulatory and inhibitory conditions have been found. Inhibition was detected after incubation of membranes under phosphorylation conditions. Stimulation, but not inhibition, requires a cytosolic factor. This factor is absent in a mutant which is unable to activate adenylate cyclase by cAMP *in vivo*. Desensitized cells have lost the stimulation of adenylate cyclase by guanine nucleotides, but retained the potency of inhibition. These results suggest that *D. discoideum* contains both Gs-like and Gi-like activities and that the activity of Gs is inhibited by phosphorylation *in vitro* and during desensitization *in vivo*.

MATERIALS AND METHODS

Materials

[2,8- 3H]cAMP (1.5 TBq/mmol) was obtained from the Amersham International; ATP, ATP[γ S], GTP p[NH]ppG, GTP[γ S], GDP and GTP[β S] were from Boehringer; cAMP, dcAMP, and dithiothreitol were from Sigma; pertussis toxin was obtained from List Biological Laboratories (Campbell, California). (Sp)-cAMP[S] was a kind gift of Drs Jastorff, Baraniak and Stec [28].

Culture conditions

Dictyostelium discoideum, wild-type strain NC-4 and mutant strain N7 [29] (kindly provided by Dr Devreotes), were grown in association with *Escherichia coli* on a buffered glucose/peptone medium [16]. Cells were harvested at the late logarithmic phase with 10 mM sodium/potassium phosphate buffer, pH 6.5 (buffer A), washed and starved in suspension in buffer A at a density of 10^7 cells/ml for 4 h.

Desensitization of adenylate cyclase

Cells were washed twice with buffer A, resuspended in this buffer at a density of 10^7 cells/ml and incubated for 15 min at 20°C in the presence or absence of 10 μ M non hydrolysable agonist (Sp)-cAMP[S]. The cell suspension was diluted 15-fold in ice-cold buffer A, and washed twice at 0°C with buffer A. The final cell pellet was resuspended in buffer B, and used for cell lysis (see below).

Incubation with pertussis toxin

Cells were starved in buffer A at a density of 10^7 cells/ml in the absence or presence of 0.2 μ g/ml pertussis toxin. After 5 h cells were washed three times in buffer A and the final cell pellet was resuspended in buffer B and used for cell lysis (see below).

Cell lysis

Cells were collected by centrifugation (2 min at $100 \times g$), washed twice with buffer A and the final cell pellet was resuspended to 2×10^8 cell/ml in buffer B (40 mM Hepes/NaOH, 5 mM EDTA, 250 mM sucrose, pH 7.7). Cells were homogenized at 0°C with mechanical force by pressing them through 3- μ m pores of a Nucleopore filter [30]; more than 99.7% of the cells were lysed. The homogenate was centrifuged at $10000 \times g$ for 5 min, the supernatant was saved and the pellet was washed twice with double the amount of buffer B. The final pellet was resuspended in buffer B containing a portion of the saved supernatant to a volume equivalent to 2×10^8 cells/ml.

Phosphorylation of membranes

Membranes were incubated at 20°C in buffer B containing 10 mM $MgCl_2$, 10 mM NaF, and 1 mM ATP[γ S]. The reaction was terminated after 5 min by addition of five volumes of ice-cold buffer B. Membranes were centrifuged, washed twice and resuspended in buffer B to the original volume.

Adenylate cyclase assay

Adenylate cyclase was measured in a total volume of 40 μ l containing buffer C (40 mM Hepes/NaOH, 3 mM EDTA, 250 mM sucrose, pH 7.7), 6 mM $MgCl_2$, 0.5 mM ATP, 10 mM dithiothreitol and 20 μ l isolated membranes (about 25 μ g protein). The incubation period was 40 min at 0°C, or 5 min at 20°C. The reaction was terminated by the addition of 10 μ l 0.1 M EDTA. Enzyme activities were destroyed by boiling the samples for 2 min; subsequently tubes were placed in ice. Under these conditions the adenylate cyclase activity in the absence and presence of guanine nucleotides is linear with time.

cAMP levels were determined by isotope-dilution assay [31]. To the tubes were added 30 μ l [3H]cAMP (18000 cpm) and 30 μ l cAMP-binding protein; both were dissolved in buffer D (150 mM potassium phosphate, 10 mM EDTA, 1 mg/ml bovine serum albumin, 3 mM sodium azide, pH 7.0). After an incubation period of 1.5 h, 60 μ l suspension containing 50 mg/ml charcoal in buffer D was added. After 1 min tubes were centrifuged at $10000 \times g$ for 2 min, and the radioactivity in 120 μ l supernatant was determined. Typical data are for boiled enzyme 8531 ± 201 cpm, boiled enzyme with 1 pmol authentic cAMP 4663 ± 126 cpm, boiled enzyme with excess cAMP 121 ± 23 cpm, and incubation at 0°C 6148 ± 189 cpm. The standard deviation of the adenylate cyclase assay at 0°C was 6% ($n = 5$); incubations were in triplicate and were performed with at least three different enzyme preparations.

The following controls were performed. The addition of EDTA effectively blocked adenylate cyclase activity (Fig. 1A), and the product that is detected in the isotope-dilution assay cochromatographs with authentic cAMP and is degraded by cyclic-nucleotide phosphodiesterase (Fig. 1B). Degradation of 0.5 mM ATP or 0.1 mM GDP, GTP, GDP[β S] and GTP[γ S] was less than 20% after an incubation period of 40 min at 0°C, or 5 min at 20°C. Degradation of cAMP during the adenylate cyclase assay was measured by adding 40000 cpm [3H]cAMP to the incubation mixture and analysis of the [3H]cAMP formed; degradation was 10% at 0°C and 3% at 20°C. Finally the effect of the additives such as GTP[γ S] on this residual phosphodiesterase activity and on the sensitivity of the isotope-dilution assay was investigated. No

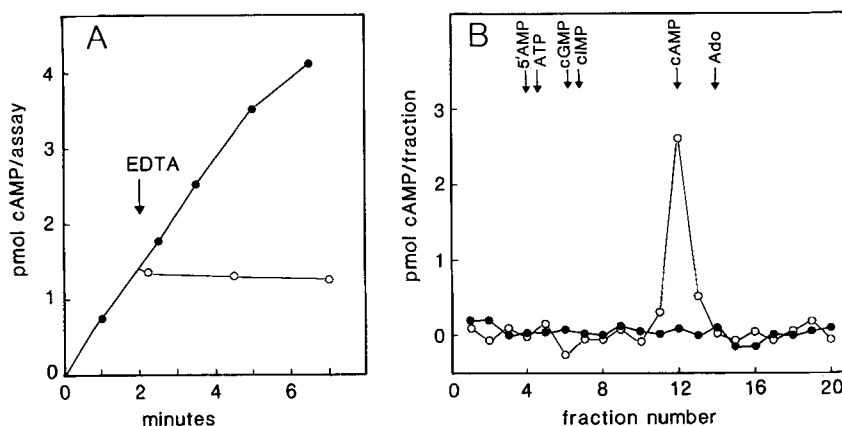


Fig. 1. Termination of the adenylate cyclase reaction by EDTA, and chromatography of the reaction product. (A) Membranes from *D. discoideum* were incubated with Mg/ATP at 20°C (●). Samples were boiled at the times indicated. EDTA (17 mM excess to Mg) was added to a part of the samples (○). cAMP levels were determined by isotope-dilution assay [31]. (B) Membranes were incubated for 5 min with Mg/ATP at 20°C. One sample was incubated with beef heart phosphodiesterase (●), and another was not (○). Both samples were chromatographed by high-performance liquid chromatography on LiChrosorb RP-18 in 10% methanol, 1 mM phosphate buffer, pH 6.5. Fractions of 0.5 ml were lyophilized, and the cAMP content was measured by isotope-dilution assay. The recovery of cAMP in the sample not treated with phosphodiesterase was more than 90%

effects were observed. The degradation of nucleotides was measured by reversed-phase high-performance liquid chromatography, using LiChrosorb RP-18. The mobile phase for the analysis of cyclic nucleotides was 1 mM phosphate buffer, 12% methanol, pH 6.5. The mobile phase for the analysis of nucleotide polyphosphates was 0.1 M potassium phosphate, 5 mM tributylamine, 10% methanol, pH 6.5.

RESULTS

Adenylate cyclase activity at 0°C and 20°C

Stimulation of *D. discoideum* cells results in the activation of adenylate cyclase; this process is relatively independent of the temperature. *In vivo* cAMP levels increase about 2-fold slower at 0°C than at 20°C [32]. In contrast, adenylate cyclase activity *in vitro* with Mg/ATP as substrate is strongly temperature-dependent with a 70-fold difference at the indicated temperatures [33] (and Table 1). It was also observed that the temperature sensitivity depends on the substrate; enzyme activity differs only 7-fold with Mn/ATP. In vertebrates adenylate cyclase is regulated by guanine nucleotide-regulatory proteins, which stimulate (Gs), or inhibit (Gi) adenylate cyclase [21]. It has been demonstrated that the sensitivity of adenylate cyclase for guanine nucleotides is lost at low concentrations of Mn^{2+} or high concentrations of Mg^{2+} [34, 35]. The assumption that adenylate cyclase in membranes of *D. discoideum* is activated by Gs at low concentrations of Mg^{2+} at 20°C but not at 0°C would explain the results of the differential temperature sensitivity with Mg/ATP and Mn/ATP as the substrate. This hypothesis will be investigated below.

Adenylate cyclase activity with other substrates

The activity of the cell-surface cAMP receptor in *D. discoideum* is probably regulated by phosphorylation, which may play a role in desensitization of adenylate cyclase [22–24]. In addition incubation of membranes under phosphorylation conditions alters the interaction between cAMP receptor and G protein, possibly by the phosphorylation of a signal trans-

Table 1. Activity of adenylate cyclase at 0°C and 20°C with different substrates

Mg/p[NH]ppA, Mn/p[NH]ppA, Mg/p[CH₂]ppA, and Mn/p[CH₂]ppA have activities that are below the detection limit (0.25, and 0.05 pmol/min⁻¹ mg protein⁻¹ at 20°C and 0°C respectively). Adenylate cyclase activity was measured at 20°C and at 0°C in a mixture containing 40 mM Hepes, 10 mM dithiothreitol, 0.25 M sucrose, 5 mM EDTA, 8 mM MgCl₂ or MnCl₂, and 0.5 mM ATP or one of its derivatives. The incubation period was 5 min at 20°C or 40 min at 0°C. The produced cAMP was determined by isotope-dilution assay [31]. The ratio indicates the difference of enzyme activity at 20°C relative to 0°C. The results shown are the means and standard deviations of three experiments with triplicate determinations

Substrate	Activity		Ratio
	20°C	0°C	
	pmol min ⁻¹ mg protein ⁻¹		
Mg/ATP	31.5 ± 1.9	0.45 ± 0.04	70 ± 8.1
Mn/ATP	39.2 ± 1.4	5.60 ± 0.45	7 ± 0.5
Mg/ATP[γS]	11.1 ± 0.7	0.38 ± 0.02	29 ± 3.3
Mn/ATP[γS]	3.9 ± 0.4	0.56 ± 0.03	7 ± 1.0

duction component which is not the receptor [27]. Under the conditions of the adenylate cyclase assay, phosphorylation reactions will probably take place and may interfere with Gs regulation of adenylate cyclase. The adenylate cyclase assay employs unlabeled ATP as substrate. Therefore, it is possible to assay enzyme activity with other substrates which either cannot phosphorylate (e.g. p[CH]ppA) or which may phosphorylate irreversible (e.g. ATP[γS]). p[NH]ppA and p[CH]ppA were not substrates of adenylate cyclase in *D. discoideum* (Table 1), nor under the conditions which are mentioned below. Adenylate cyclase with Mg/ATP[γ-S] had 85% of the activity of Mg/ATP at 0°C, and 35% at 20°C. Finally, Mn/ATP[γ-S] had less than 15% of the activity of Mn/ATP at both temperatures. This indicates that ATP[γ-S] can act as a substrate for adenylate cyclase, but that it may have additional regulatory activities. Recently we have shown

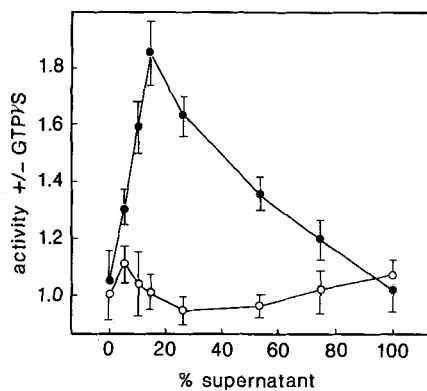


Fig. 2. Adenylate cyclase stimulation by GTP[γ S] requires a supernatant factor. Cells were lysed at a density of 2×10^8 cells/ml and the homogenate was centrifuged. The supernatant was saved and the pellet was washed twice. The final pellet was resuspended to the original membrane density in buffer with the indicated fraction of the saved supernatant. Adenylate cyclase activity was determined at 0°C (●) or at 20°C (○) in the absence and presence of 100 μ M GTP[γ S]. The results shown are the means and standard deviations of triplicate determinations of an experiment reproduced twice

that ATP[γ -S] irreversibly alters the interaction between cAMP receptor and G protein [27]. Also this aspect of adenylate cyclase regulation will be investigated below.

Adenylate cyclase stimulation by GTP[γ S] requires a cytosolic factor

When adenylate cyclase activity in the presence and absence of GTP[γ S] was measured at 0°C, stimulation by GTP[γ S] was not observed in a crude homogenate nor in washed membranes, but only in unwashed membranes. Therefore, different portions of the supernatant were re-added to the washed pellet and adenylate cyclase activity was measured at 0°C and 20°C (Fig. 2). GTP[γ S] did not alter enzyme activity at 20°C. In contrast, GTP[γ S] induced a small but significant increase in enzyme activity in the presence of a small amount of the supernatant; the ability to be activated was lost at higher amounts.

The supernatant factor was not sedimented after centrifugation at $100000 \times g$ for 1 h, was heat-labile and eluted in the void volume of a Sephadex G-50 column, indicating a molecular mass of more than 20 kDa (data not shown). The following experiment suggests that the supernatant factor is not the α subunit of a G protein. Membranes or supernatant were incubated for 15 min at 0°C with GTP[γ S]. Subsequently GTP[γ S] was removed by washing the membranes, or by passing the supernatant through a Sephadex G-25 column. GTP[γ S]-treated preparations were reconstituted with untreated preparations, and adenylate cyclase activity was measured in the absence of GTP[γ S]. It was observed that adenylate cyclase from GTP[γ S]-treated membranes was activated by the untreated supernatant. In contrast adenylate cyclase from untreated membranes was not activated by the addition of GTP[γ S]-treated supernatant.

Temperature and Mg^{2+} dependence of adenylate cyclase activation

Adenylate cyclase activity was measured in the presence of the supernatant factor at different temperatures in the

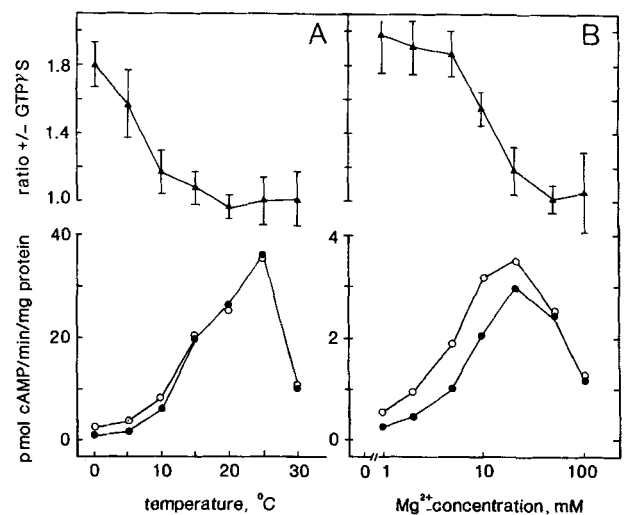


Fig. 3. Temperature and Mg^{2+} dependence of adenylate cyclase activation by GTP[γ S]. Membranes were prepared as described in the legend of Fig. 2, and contained 15% of the supernatant. Adenylate cyclase activity was measured in the absence (●) or presence (○) of 100 μ M GTP[γ S]. Their ratio is indicated by (▲). (A) Enzyme activity was determined at different temperatures with 3 mM Mg^{2+} in excess to EDTA. (B) Enzyme activity was determined at 0°C, with different concentrations of Mg^{2+} in excess of EDTA

Table 2. Adenylate cyclase activity in wild-type NC-4 and mutant N7. Wild-type NC-4 and mutant N7 cells were homogenized at a density of 2×10^8 cells/ml. The homogenates were centrifuged, the supernatants were saved, the pellets were washed twice and resuspended to the original membrane density in the absence or presence of 15% of the indicated supernatant. Adenylate cyclase was determined at 0°C in the absence or presence of 100 μ M GTP[γ S]. The enzyme activities are shown for the incubations without GTP[γ S], and are the means and standard deviations of triplicate determinations from a typical experiment. The ratios of enzyme activities with/without GTP[γ S] are shown in the last column, and are the means and standard deviations from three independent experiments

Pellet	Supernatant	Activity pmol min ⁻¹ mg protein ⁻¹	Ratio ± GTP[γ S]
NC-4	—	0.49 ± 0.04	0.97 ± 0.05
	NC-4	0.41 ± 0.04	1.83 ± 0.20
	N7	0.43 ± 0.02	0.95 ± 0.09
N7	—	0.32 ± 0.03	1.05 ± 0.10
	NC-4	0.26 ± 0.01	1.80 ± 0.07
	N7	0.27 ± 0.03	1.12 ± 0.17

presence and absence of GTP[γ S] (Fig. 3). Enzyme activity increases about 70-fold between 0°C and 25°C. Activation by GTP[γ S] was only detectable at temperatures below 10°C. Optimal stimulation was at 0°C.

Adenylate cyclase activity strongly depends on the Mg^{2+} concentration (Fig. 3B). The optimal concentration at 0°C is 20 mM; however, stimulation by GTP[γ S] was almost absent at this high concentration. Stimulation by GTP[γ S] was approximately 2-fold at Mg^{2+} concentrations below 5 mM. The optimum of activity and ability to be activated of adenylate cyclase is at 0°C and with 3 mM Mg^{2+} .

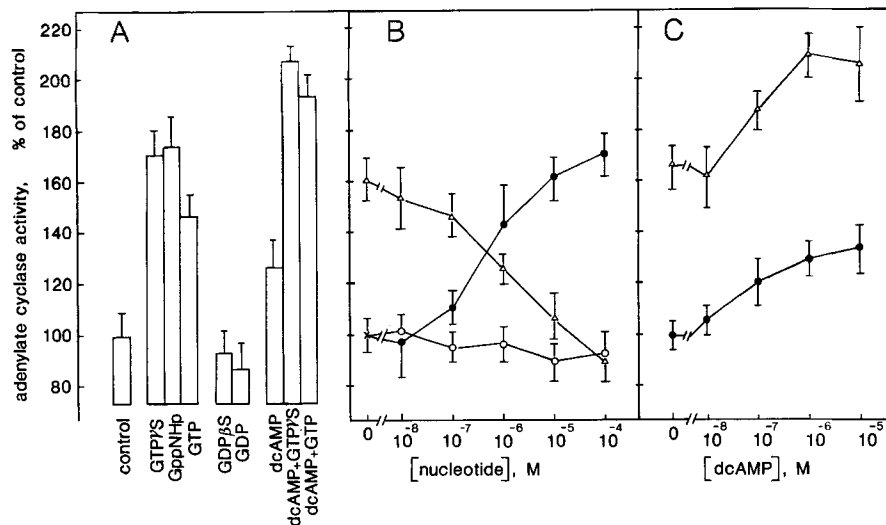


Fig. 4. Nucleotide specificity of adenylate cyclase stimulation. Membranes were prepared as described in the legend of Fig. 2, and contained 15% of the supernatant. (A) Adenylate cyclase activity was determined in the presence of guanine nucleotides (30 μ M) or dcAMP (1 μ M). (B) Adenylate cyclase activity was determined in the presence of different concentrations guanine nucleotides. (●), GTP[γ S]; (○), GDP[β S]; (△), GDP[β S] with 10 μ M GTP[γ S]. (C) Adenylate cyclase activity was measured in the presence of different concentrations dcAMP with (△) or without (●) 10 μ M GTP[γ S]. The results shown are the means and standard deviations of three independent experiments

A mutant with defects in the cytosolic factor

Recently we characterized a mutant, N7, which showed defects in the stimulation of adenylate cyclase by cAMP *in vivo*. This mutant had normal levels of adenylate cyclase, and its cAMP receptors were regulated by guanine nucleotides as in wild-type cells (unpublished results). Table 2 shows that adenylate cyclase activity in mutant as well as wild-type membranes was not stimulated in the presence of the supernatant of the mutant, while both could be activated in the presence of the supernatant of the wild type. This indicates that the defect of the mutant is probably located at the cytosolic cofactor that is required for guanine-nucleotides-mediated activation of adenylate cyclase in *D. discoideum*.

Nucleotide specificity for stimulation of adenylate cyclase

Adenylate cyclase activity was measured under stimulation conditions in the presence of different concentrations of nucleotides. GTP, GTP[γ S], and p[NH]ppG (30 μ M) all stimulated adenylate cyclase activity. Guanosine diphosphates at the same concentration did not stimulate, and even slightly inhibited adenylate cyclase (Fig. 4A). dcAMP (1 μ M) stimulated adenylate cyclase 1.3-fold and potentiated the stimulation by GTP[γ S] and GTP with the same factor. Half-maximal effects of dcAMP were observed at 100 nM (Fig. 4C), which is similar to the binding affinity of the cell-surface receptor [36]. The dose-response curve for stimulation by GTP[γ S] is shown in Fig. 4B. Half-maximal stimulation occurred at about 1 μ M GTP[γ S]. GDP[β S] did not activate adenylate cyclase, and antagonized the stimulation by GTP[γ S]; half-maximal antagonism occurred at about 1 μ M GDP[β S].

Inhibition of adenylate cyclase by GTP[γ S] after preincubation of membranes with ATP[γ S]

Previously we have shown that a preincubation of membranes with Mg/ATP[γ S] results in diminished inhibition of cAMP binding by guanine nucleotides, presumably by

Table 3. Adenylate cyclase activity in membranes treated with Mg/ATP[γ S]

Cells were lysed and the homogenate was centrifuged. The supernatant was saved, the pellet was washed twice, and resuspended to the original volume in the absence or presence of 15% supernatant (control). A portion of the washed pellet was incubated with 10 mM NaF, 10 mM MgCl₂, and 1 mM ATP[γ S] for 5 min at 20°C, washed twice at 0°C, and the final pellet was resuspended to the original volume in the absence or presence of 15% supernatant (ATP[γ S]). Adenylate cyclase activity was determined at 0°C in the absence or presence of 100 μ M GTP[γ S]. The activity and ratio \pm [GTP[γ S]] are defined as described in Table 2

Pellet	Supernatant	Activity pmol min ⁻¹ mg protein ⁻¹	Ratio \pm GTP[γ S]
Control	—	0.59 \pm 0.05	1.05 \pm 0.10
	+	0.49 \pm 0.03	1.87 \pm 0.21
ATP[γ S]	—	0.53 \pm 0.04	0.73 \pm 0.05
	+	0.50 \pm 0.02	0.70 \pm 0.03

phosphorylation of one of the components involved in signal transduction [27]. Table 3 summarizes the effect of a preincubation of membranes with Mg/ATP[γ S] on adenylate cyclase activity. Before the preincubation with Mg/ATP[γ S] the enzyme was activated by GTP[γ S] in the presence of the supernatant factor, whereas the enzyme was inhibited by GTP[γ S] after preincubation of membranes with Mg/ATP[γ S]. This inhibition was not affected by the supernatant factor. A preincubation with Mg/p[CH]ppA or Mg/p[NH]ppA had no effect (data not shown), supporting the hypothesis that the effect of Mg/ATP[γ S] is mediated by a phosphorylation reaction.

Pertussis toxin prevents adenylate cyclase inhibition by GTP[γ S]

In vertebrates, inhibition of adenylate cyclase by guanine nucleotides is mediated by Gi. Pertussis toxin causes the ADP-

Table 4. Adenylate cyclase activity in pertussis-toxin-treated cells

Cells were starved for 5 h in the absence or presence of 0.2 µg/ml pertussis toxin (PT). Cells were harvested, washed, lysed, and the homogenate was centrifuged. The supernatants were saved, the pellets were washed twice and resuspended to the original membrane density in the presence of absence of 15% of the indicated supernatants. Washed pellets were also incubated with Mg/ATP[γS] for 5 min at 20°C, washed and resuspended to the original membrane density. Adenylate cyclase was measured at 0°C in the absence or presence of 100 µM GTP[γS]. The activity and the activation ratio are defined in Table 2

Pellet	Supernatant	Activity pmol min ⁻¹ mg protein ⁻¹	Ratio ± GTP[γS]
Control	—	0.66 ± 0.02	0.99 ± 0.08
	control	0.53 ± 0.04	1.73 ± 0.11
	PT	0.58 ± 0.05	1.74 ± 0.12
PT	—	0.81 ± 0.06	1.05 ± 0.14
	control	0.65 ± 0.02	1.64 ± 0.12
	PT	0.62 ± 0.04	1.93 ± 0.24
Control ATP[γS]	—	0.48 ± 0.03	0.71 ± 0.05
PT/ATP[γS]	—	0.56 ± 0.06	1.06 ± 0.07

ribosylation, and thereby inhibition, of Gi [21]. The nature of GTP[γS]-mediated inhibition of adenylate cyclase in *D. discoideum* was investigated in membranes derived from pertussis-toxin-treated cells (Table 4). Adenylate cyclase of control cells was regulated as described before: stimulation by GTP[γS] in the presence of the supernatant factor, and inhibition by GTP[γS] after treatment of membranes with ATP[γS]. Pertussis toxin did not alter the properties of stimulation of adenylate cyclase, neither in the pellet nor in the supernatant fraction. In contrast, inhibition of adenylate cyclase activity by GTP[γS] was no longer observed in membranes derived from toxin-treated cells.

Nucleotide specificity of inhibition of adenylate cyclase

The effects of nucleotides on adenylate cyclase activity in membranes with ATP[γS] are shown in Fig. 5. GTP, GTP[γS], and p[NH]ppG (30 µM) inhibited adenylate cyclase to a similar extent. GDP and GDP[βS] are not inhibitory at this concentration. dcAMP slightly reduced adenylate cyclase activity in the absence or presence of GTP[γS], but the effect was statistically not significant (Fig. 5A). Inhibition of adenylate cyclase by GTP[γS] was maximally 30% (Fig. 5B); half-maximal inhibition occurred at about 1 µM. GDP[βS] did not inhibit adenylate cyclase up to 100 µM and antagonized the inhibition by GTP[γS] with a half-maximal effect at about 1 µM GDP[βS].

Adenylate cyclase in desensitized cells

In vivo cAMP activates adenylate cyclase but also induces desensitization of this enzyme to further stimulation. Membranes were prepared from control and desensitized cells, and adenylate cyclase activity was measured under stimulatory and inhibitory conditions (Table 5). Adenylate cyclase in control membranes was stimulated by GTP[γS] when reconstituted with the supernatant obtained from either control or desensitized cells. In contrast adenylate cyclase in membranes from desensitized cells was not stimulated by

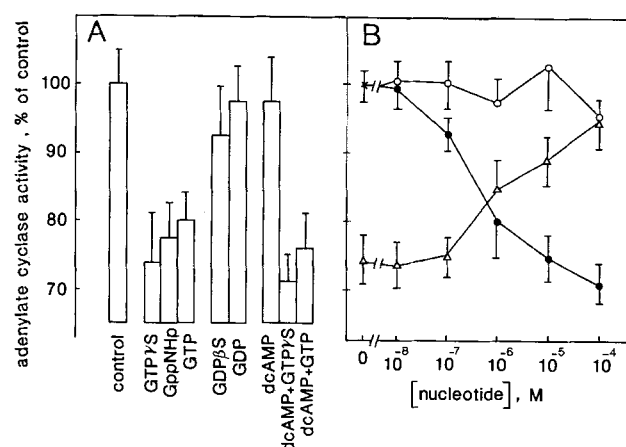


Fig. 5. Nucleotide specificity of adenylate cyclase inhibition. Isolated membranes were preincubated with Mg/ATP[γS] for 5 min at 20°C, and washed extensively. (A) Adenylate cyclase activity was measured in the presence of guanine nucleotides (30 µM), or dcAMP (1 µM). (B) Adenylate cyclase activity was determined in the presence of different concentrations guanine nucleotides. (●), GTP[γS]; (○), GDP[βS]; (△), GDP[βS] with 10 µM GTP[γS]. The results shown are the means and standard deviations of three independent experiments

Table 5. Adenylate cyclase in desensitized cells

Cells were starved for 4 h, washed and incubated at 20°C for 15 min with 10 µM (Sp)-cAMP[S], which induced desensitization of adenylate cyclase stimulation *in vivo* (desensitized). Control cells were not incubated with (Sp)-cAMP[S]. Cells were then washed at 0°C, lysed, and the homogenates were centrifuged. The supernatants were saved, the pellets were washed twice and resuspended to the original membrane density in the presence of 15% of the indicated supernatants. Washed pellets were also incubated with Mg/ATP[γS] for 5 min at 20°C, washed and resuspended to the original membrane density. Adenylate cyclase was measured in the absence or presence of 100 µM GTP[γS]. The activity and the activation ratio are defined in Table 2

Pellet	Supernatant	Activity pmol min ⁻¹ mg protein ⁻¹	Ratio ± GTP[γS]
Control	—	0.58 ± 0.03	1.15 ± 0.09
	control	0.50 ± 0.02	1.73 ± 0.14
	desensitized	0.53 ± 0.05	1.85 ± 0.12
Desensitized	—	0.59 ± 0.03	0.97 ± 0.06
	control	0.57 ± 0.03	0.96 ± 0.04
	desensitized	0.60 ± 0.06	1.00 ± 0.09
Control/ATP[γS]	—	0.50 ± 0.03	0.77 ± 0.05
Desensitized/ATP[γS]	—	0.54 ± 0.08	0.67 ± 0.03

GTP[γS] in the presence of supernatant from control as well as desensitized cells. This indicates that the supernatant factor is probably not altered during desensitization and that the loss of stimulation is localized in the membrane fraction.

Membranes from control and desensitized cells were also incubated under phosphorylation conditions for the assay of inhibition of adenylate cyclase by GTP[γS]. Apparently inhibition is not lost in membranes from desensitized cells, and is slightly more pronounced than in control membranes; the difference is significant at a level of 5%. This suggests that desensitization of adenylate cyclase by cAMP *in vivo* results from the alteration of a signal transduction component in the membrane fraction, by which stimulation but not inhibition of adenylate cyclase by guanine nucleotides *in vitro* is lost.

DISCUSSION

cAMP binds to cell-surface cAMP receptors and induces the activation of adenylate cyclase in *D. discoideum* cells. Binding of cAMP to isolated membranes is modulated by guanine nucleotides, suggesting an interaction between cAMP receptor and a guanine-nucleotide-regulatory protein (G protein). The existence of such a protein has been suggested previously by demonstrating that *D. discoideum* cells contain a 42-kDa protein, which binds GTP and can be ADP-ribosylated with cholera toxin [20]. Here we report on the regulation of adenylate cyclase *in vitro* by guanine nucleotides, cAMP-receptor agonist, phosphorylation conditions and pertussis toxin in desensitized, mutant and control cells.

Adenylate cyclase activity was localized in the membranes. Stimulation by GTP[γ S] was observed only at 0°C in the presence of a small amount of the supernatant. The absence of stimulation at 20°C could be related to the observation of the temperature sensitivities of adenylate cyclase with Mg/ATP or Mn/ATP as the substrate (Table 1). Adenylate cyclase is 70-fold less active at 0°C than at 20°C with Mg/ATP, and 7-fold less active with Mn/ATP as the substrate. This could be explained by the hypothesis that adenylate cyclase is already activated at 20°C in the presence of Mg/ATP or Mn/ATP, and at 0°C in the presence of Mn/ATP; the enzyme is not activated at 0°C in the presence of Mg/ATP. Thus, stimulation of adenylate cyclase activity by guanine nucleotides could only be observed at 0°C with Mg/ATP as the substrate.

Stimulation of adenylate cyclase by guanine nucleotides is specific for guanosine triphosphates. Guanosine diphosphates are not stimulatory and antagonize the stimulation by guanosine triphosphates. The cAMP-receptor agonist, dcAMP, potentiates the stimulation 1.3-fold, and has a half-maximal effect at 100 nM, which is similar to its affinity for the cell-surface cAMP receptor in *D. discoideum* [36]. These observations suggest that adenylate cyclase in *D. discoideum* membranes is regulated by a protein with similar characteristics as the vertebrate stimulatory guanine-nucleotide-regulatory protein, Gs. This observation agrees well with previous results that cAMP induced a transition of high-affinity to low-affinity binding of cAMP to *D. discoideum* cells [15]. This affinity transition was induced in membranes by guanine nucleotides [17] and has been associated in vertebrates with the activation of Gs [37].

cAMP not only induces a rapid alteration of binding affinity of the receptor, but also the slower alteration of the electrophoretic mobility of the photoaffinity-labelled cAMP receptor [22]. The change of electrophoretic mobility is probably due to receptor phosphorylation [23] and has been associated with the desensitization of cAMP-mediated stimulation of adenylate cyclase [24]. Recently we observed that preincubation of *D. discoideum* membranes under phosphorylation conditions resulted in the alteration of the interaction between cAMP receptor and G protein [27]. This alteration was reversible with Mg/ATP and irreversible with Mg/ATP[γ S] as substrate of the putative endogenous protein kinase. It has been shown that ATP[γ S] is a substrate of many protein kinases, but that the protein phosphothioate is not easily hydrolysed by phosphatases (reviewed in [38]). Therefore, membranes were preincubated with Mg/ATP[γ S], and the effect on the stimulation of adenylate cyclase by GTP[γ S] was measured. Stimulation of adenylate cyclase by GTP[γ S] was converted into inhibition after treatment of membranes with Mg/ATP[γ S]. Inhibition was specific for guanosine triphosphates and antagonized by guanine

diphosphates. In addition, inhibition was no longer observed in membranes derived from pertussis-toxin-treated cells. In vertebrates pertussis toxin catalyses the ADP-ribosylation of a specific protein, Gi, and blocks the inhibition of adenylate cyclase by GTP [21]. In vertebrates, however, pertussis toxin does not block the GTP[γ S]-mediated inhibition of adenylate cyclase [39]. These observations suggest that *D. discoideum* membranes contain both Gs-like and Gi-like activities; and that these activities can be modulated by phosphorylation conditions.

It was already mentioned that cAMP induces the phosphorylation of the cAMP receptor and that this could be the molecular mechanism of desensitization of adenylate cyclase in *D. discoideum* [24]. The present observation that preincubation of membranes under phosphorylation conditions shifted the regulation of adenylate cyclase by guanine nucleotides from stimulation to inhibition led us to investigate the regulation of adenylate cyclase in desensitized cells. We observed that adenylate cyclase in desensitized cells could no longer be stimulated by guanine nucleotides, while inhibition by guanine nucleotides in phosphorylated membranes was maintained. This allows for the possibility of the dual regulation of adenylate cyclase in *D. discoideum* by Gs-like and Gi-like activities, which is modulated by phosphorylation of the cAMP receptor. It should be noted, however, that we have not been able to show that the receptor is phosphorylated after the incubations of membranes with ATP[γ S] [27]. Therefore, the relationship between the observations with phosphorylated membranes and with membranes from desensitized cells requires further investigations. The recent observation that pertussis toxin inhibits desensitization of adenylate cyclase stimulation (unpublished results) may help to elucidate the mechanism of desensitization in *D. discoideum*.

Stimulation of adenylate cyclase in washed membranes of *D. discoideum* requires a factor that is present in the supernatant of a crude homogenate. This factor is heat-labile, and has a molecular mass above 20 kDa. The observation that this factor is absent in a mutant N7, which is unable to activate adenylate cyclase with cAMP *in vivo*, strongly suggests that this factor is an essential component for adenylate cyclase regulation in *D. discoideum*. It is unlikely that this factor is Gs, since GTP[γ S]-mediated stimulation is associated with the particulate and not with the cytosol fraction of a cell homogenate. Experiments are in progress to reveal the identity of this factor and the possible presence in vertebrate cells.

Although the qualitative properties of adenylate cyclase regulation in *D. discoideum* membranes are pronounced and reproducible, the quantitative data are not very impressive. Activation of adenylate cyclase *in vitro* was never more than 2-fold. It can be estimated that activation *in vivo* by cAMP is at least 6-fold [40]. Several possibilities could account for the small stimulation *in vitro*. First, phosphorylation alters the regulation of adenylate cyclase. The incubation mixture contains Mg/ATP; therefore phosphorylation is not prevented in the present experiments. Unfortunately, p[NH]ppA and p[CH₂]ppA, which are unable to phosphorylate, are not a substrate for adenylate cyclase from *D. discoideum*. Second, the simultaneous presence and activation of Gs and Gi could obscure stronger stimulation. Third, stimulation could not be observed at 20°C, possibly because of the activation of the guanine-nucleotide-regulatory proteins by low concentrations of Mg²⁺. The possibility cannot be excluded that partial activation occurs also at 0°C. Finally the experiment which indicates the requirement for a cytosolic factor (Fig. 2) shows

that activation of adenylate cyclase diminishes at higher concentrations of the supernatants. The elimination of this inhibitory component may potentiate the extent of stimulation by guanine nucleotides.

Although the properties of adenylate cyclase from *D. discoideum* and vertebrates may differ considerably, their regulation has many properties in common. The agonist binds to cell-surface receptors, which can exist in a high and low-affinity state. An interconversion of binding states occurs during the time period in which adenylate cyclase is activated. In membranes, guanine nucleotides promote the transition of high-affinity to low-affinity receptors. Many cells contain both Gs and Gi. Phosphorylation of the receptor diminishes Gs-mediated stimulation of adenylate cyclase. The differential activation of Gs and Gi that depends on the phosphorylation state of the receptor or another transduction component could be a general mechanism of adenylate cyclase desensitization.

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